Pirfenidone inhibits macrophage infiltration in 5/6 nephrectomized rats

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Chen JF, Ni HF, Pan MM, Liu H, Xu M, Zhang MH, Liu BC. Pirfenidone inhibits macrophage infiltration in 5/6 nephrectomized rats. Am J Physiol Renal Physiol 304: F676–F685, 2013. First published November 14, 2012; doi:10.1152/ajprenal.00507.2012.—Tubulointerstitial macrophage infiltration is a hallmark of chronic kidney disease involved in the progression of renal fibrosis. Pirfenidone is a newly identified antifibrotic drug, the potential mechanism of which remains unclear. The aim of this study was to investigate the effects of pirfenidone on M1/M2 macrophage infiltration in nephrectomized rats. Nephrectomized rats were treated with pirfenidone by gavage for 12 wk. Twenty-four hour urinary protein, N-acetyl-B-

glycosaminidase (NAG) activity, systolic blood pressure, and C-reactive protein were determined. Parafin-embedded sections were stained for CD68, CCR7, and CD163 macrophages. Monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1a (MIP-1a), as well as M1 and M2 macrophages secretory markers, were evaluated by real-time RT-PCR and Western blotting analysis. Pirfenidone significantly improved the elevated proteinuria and NAG activity, systolic blood pressure, and C-reactive protein. Pirfenidone significantly decreased the infiltrating macrophages. The number of M1 and M2 macrophages was significantly lower after pirfenidone treatment. MCP-1 and MIP-1a were increased in nephrectomized rats at mRNA and protein levels. Pirfenidone treatment significantly inhibited their expression. The TNF-α, IL-6, and nitric oxide synthases-2 expressed by M1 macrophages were increased in nephrectomized rats, and pirfenidone significantly attenuated their expression. Pirfenidone treatment also significantly decreased arginase-1, dectin-1, CD206, and CD86 expressed by M2 macrophages. Thus pirfenidone inhibits M1 and M2 macrophage infiltration in 5/6 nephrectomized rats, which suggests its efficacy in the early and late periods of renal fibrosis.

RENAL FIBROSIS REPRESENTS an important final common pathway for the progression of renal diseases, including chronic glomerulonephritis, diabetic nephropathy, and chronic renal allograft injury, which often result in chronic kidney disease (CKD; Ref. 11). In addition to excessive extracellular matrix deposition, renal fibrosis is commonly associated with inflammatory cell infiltration, myofibroblast accumulation, tubular atrophy, and loss of peritubular or glomerular capillaries, during which a sustained inflammatory process may result in greater tissue damage and eventually lead to fibrogenesis. Thus anti-inflammatory therapy needs to be investigated in the treatment of renal fibrosis.

Macrophage infiltration is commonly implicated in CKD. Macrophages comprise a heterogeneous population of cells with diverse functions and phenotypic plasticity. Their pathogenic role in renal inflammation and fibrosis is commonly recognized. Macrophages can generally be divided into two groups: M1 (classically activated) and M2 (alternatively activated) macrophages (21). M1 macrophages exhibit proinflammatory properties, whereas, M2 macrophages promote fibrogenesis and tissue remodeling. Studies have shown that inhibition of macrophage infiltration reduces proteinuria and structural injury in models including antiglomerular basement disease, puromycin aminonucleoside nephrosis, and Heymann nephritis (8, 31, 41). It has been suggested that macrophage inhibition is likely to be an effective therapeutic approach in CKD treatment.

Pirfenidone [5-methyl-1-phenyl-2-(1H)-pyridone] is an oral derivative of pyridine, which was initially developed as an antihelminthic and antipyretic agent. Since its discovery as an antifibrotic agent in a hamster model of bleomycin-induced pulmonary fibrosis (13), pirfenidone has been clinically evaluated for its safety and efficacy in numerous disorders including idiopathic pulmonary fibrosis (27), multiple sclerosis (40), chronic hepatitis C (2), chronic allograft rejection (23), and fibrotic renal diseases (4, 30). Although the precise mechanism of pirfenidone is incompletely understood, it exhibits both anti-inflammatory and antifibrotic effects. Less attention has been focused on its anti-inflammatory effects. In a rat lung transplant model, pirfenidone limited the alloimmune/inflammatory response, as reflected in the reduction of intraallograft neutrophil and lymphocyte recruitment (16). Iyer et al. (12) also noted that pirfenidone suppressed the belomycin-induced increased influx of neutrophils, lymphocytes, and macrophages (12).

In this study, we investigated the protective effects of pirfenidone in a 5/6 nephrectomized (SNx) rat model. Specially, the influence of pirfenidone on the infiltration of M1 and M2 macrophages and their inflammatory mediators was studied. Our study provided a novel mechanism of pirfenidone action in the treatment of renal fibrosis.

MATERIALS AND METHODS

Animals. Six-week-old male Sprague-Dawley (SD) rats weighing 180–200 g were purchased from Shanghai Slac Laboratory Animal (Shanghai, China). Rats were maintained under stable room temperature and regular 12-h dark and light rhythm for 1 wk before the start of experiment. They were fed standard rat chow and had free access to tap water. For urine collection, each rat was placed in a metabolic cage for 24 h. All animals received humane care in compliance with the university guidelines. The experimental protocol was approved by the Ethics Review Committees for Animal Experimentation of Southeast University.

Surgical procedure and drug administration. Each rat was anesthetized with chloral hydrate solution at a dose of 33 mg/100 g via intraperitoneal injection. Twenty rats underwent a 5/6 nephrectomy, in which the right kidney was removed, followed by the ablation of two-thirds of the left kidney. A sham operation was performed on 10 additional rats for use as a non-nephrectomized control (sham).

Pirfenidone (Licheng Chemical, Shanghai, China) was suspended in a 0.5% carboxymethylcellulose solution (vehicle). Nephrectomized rats were randomly divided into two groups: a without treatment group and a treated group. The treated group received pirfenidone (50 mg/kg body wt) by gavage for 12 wk. Twenty-four hour urinary protein, N-acetyl-B-glycosaminidase (NAG) activity, systolic blood pressure, and C-reactive protein were determined. Parafin-embedded sections were stained for CD68, CCR7, and CD163 macrophages. Monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1a (MIP-1a), as well as M1 and M2 macrophages secretory markers, were evaluated by real-time RT-PCR and Western blotting analysis. Pirfenidone significantly improved the elevated proteinuria and NAG activity, systolic blood pressure, and C-reactive protein. Pirfenidone significantly decreased the infiltrating macrophages. The number of M1 and M2 macrophages was significantly lower after pirfenidone treatment. MCP-1 and MIP-1a were increased in nephrectomized rats at mRNA and protein levels. Pirfenidone treatment significantly inhibited their expression. The TNF-α, IL-6, and nitric oxide synthases-2 expressed by M1 macrophages were increased in nephrectomized rats, and pirfenidone significantly attenuated their expression. Pirfenidone treatment also significantly decreased arginase-1, dectin-1, CD206, and CD86 expressed by M2 macrophages. Thus pirfenidone inhibits M1 and M2 macrophage infiltration in 5/6 nephrectomized rats, which suggests its efficacy in the early and late periods of renal fibrosis.

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PIRFENIDONE INHIBITS MACROPHAGE INFILTRATION

Table 1. Primer sequences for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>NM_031530</td>
<td>AGCATCCACGCTGCTTCTGCTGAGTGA</td>
<td>GATCATCTTGGCAGTAAGTGG</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>NM_013025</td>
<td>GGCCTGCTGAGAACGACTGTTGAG</td>
<td>GAATTTGCGGCTACATAGCG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>NM_012675</td>
<td>TGGCTGCCTGAGGGTCAGAGCTG</td>
<td>GTCGTCGCGTGGAGAACGACTGG</td>
</tr>
<tr>
<td>IL-6</td>
<td>NM_012589</td>
<td>GCTCCTCGAGGAAAGGCTAGTG</td>
<td>GAGGTGGCCGTGCAAAACA</td>
</tr>
<tr>
<td>Arginase-1</td>
<td>NM_017134</td>
<td>CCGAGCAATTAAGAAGAGC</td>
<td>CCGGTGCTCTGCACAGTG</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>NM_00173386</td>
<td>CAGATGAGTGGACGAGAGTGAG</td>
<td>TTGGTGTCTTCTTTCTTTGAGG</td>
</tr>
<tr>
<td>CD206</td>
<td>NM_00106123</td>
<td>AACTGCGTTGGATGGAAGG</td>
<td>TAAACCAGCAGTTGCTGCAGA</td>
</tr>
<tr>
<td>CD86</td>
<td>NM_020081</td>
<td>GAACACAGCAAGAGTCAATTA</td>
<td>GCCGTCTCATTACTGAAATTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>TGTGTGCAAGAGAGGAGGTTG</td>
<td>GTCGAGAAGATACACTGCA</td>
</tr>
</tbody>
</table>

MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α.

(control, n = 10) and a group treated with pirfenidone (n = 10). The volume of administration was determined by body weight. Pirfenidone (500 mg·kg⁻¹·day⁻¹) was administered orally once daily for 12 wk until death, and the vehicle was used as a control in the nephrectomized rats without treatment and the sham group.

Body weight, 24-h urinary protein and N-acetyl-β-D-glucosaminidase (NAG) activity were determined in alternating week. Systolic blood pressure (SBP) was measured using a tail cuff and a pneumatic pulse transducer (RBP-1B). Twelve weeks after the operation, all animals were killed, and the partial kidneys were collected and weighed. The renal tissue was immersed in 10% buffered formalin for histological evaluation, and the remaining tissue was frozen with liquid nitrogen and stored at −80°C until analysis via Western blotting. Serum creatinine, blood urea nitrogen, and serum C-reactive protein (CRP) were also determined after serum collection.

Biochemical analysis. For total protein measurement, a biuret assay method was used to determine the 24-h urinary protein. Serum concentrations of urea nitrogen and creatinine were determined by automatic analyzers (Hitachi, Tokyo, Japan). NAG activity was determined by an assay kit (Jiancheng, Nanjing, China), and serum CRP was determined by a rat ELISA kit (BD).

Histological studies. Renal tissue fixed in 10% buffered formalin was embedded in paraffin. Sections 2- to 4-μm thick were stained with periodic acid–Schiff reagent and Masson’s trichrome stain and with periodic acid–Schiff reagent and Masson’s trichrome stain and with periodic acid–Schiff reagent and Masson’s trichrome stain and with periodic acid–Schiff reagent and Masson’s trichrome stain and with periodic acid–Schiff reagent and Masson’s trichrome stain and with periodic acid–Schiff reagent and Masson’s trichrome stain.

Immunohistochemical analysis. For analysis of renal inflammatory cell infiltration, paraffin-embedded sections were immunohistochemically stained for CD68-positive macrophages and CCR7-(M1 macrophages)-positive and CD163-(M2 macrophages)-positive cells. Paraffin-embedded tissues were incubated with primary rat anti-CD68 (Santa Cruz Biotechnology), anti-CCR7 (Epitomics, Burlingame, CA), and anti-CD163 (Santa Cruz Biotechnology) antibodies, followed by appropriate secondary antibodies incubation. Diaminobenzidine was used for visualization of immunoreactivity, followed by hematoxylin for nuclear counterstaining. The numbers of CD68-, CCR7-, and CD163-positive cells were evaluated by counting the infiltrating cells in at least 20 randomly selected tubulointerstitial and glomerular sections.

Quantitative real-time RT-PCR. Total RNA of kidney cortex was extracted by Trizol reagent (Invitrogen). cDNA was synthesized using a reverse transcription system kit (Takara). Real-time RT-PCR was used to determine monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), TNF-α, IL-6, nitric oxide synthases-2 (iNOS), arginase-1, dectin-1, CD206, and CD86 gene expression. Reactions were performed on ABI PRISM 7300 real-time PCR system (Applied Biosystems, CA). Primer sequences were as listed in Table 1. GAPDH was served as a control for target genes for reaction efficiency. Results were analyzed using the comparative cycle threshold (ΔΔCT) method.

Western blotting. Proteins from the kidney cortex extracts were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore). Nonspecific antibody binding was blocked by a preincubation of the membranes in 1× TBS containing 5% skim milk for 1 h at room temperature. The membranes were then incubated overnight at 4°C with the primary antibodies against transforming growth factor-β1(TGF-β1), connective tissue growth factor (CTGF), α-smooth muscle actin (α-SMA), fibronectin, fibroblast-specific protein-1 (FSP-1), MCP-1, MIP-1α, TNF-α, IL-6, iNOS, arginase-1, dectin-1, CD206, and CD86 followed by incubation with the appropriate peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, the signals were detected using an ECL advanced system (GE Healthcare, Chalfont St. Giles, UK).

Correlation analysis. Correlations between macrophage infiltration and glomerulosclerosis/tubulointerstitial fibrosis after pirfenidone treatment were calculated using the Spearman rank-order correlation. P < 0.05 was considered to be statistically significant.

Statistical analysis. All results are expressed as the means ± SE. The comparisons among the different groups were made by one-way ANOVA using SPSS 17.0 software. Differences with P values <0.05 were considered significant.

RESULTS

Effects of pirfenidone on renal and systemic parameters in SNx rats. As shown in Table 2, although all animal groups had a similar body weight at the beginning of the experiment, the sham-operated group had gained more weight than the SNx rats by the end of the experiment. In addition, the weight of the one-sided kidney relative to body weight increased in the SNx rats.

Table 2. Influence of pirfenidone on the general parameters in experimental animals

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BW, g</th>
<th>KW/BW, g/kg</th>
<th>CRP, mg/l</th>
<th>SCR, μmol/l</th>
<th>BUN, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>10</td>
<td>541 ± 37</td>
<td>2.99 ± 0.12</td>
<td>1.47 ± 0.21</td>
<td>42.20 ± 6.81</td>
<td>9.58 ± 0.45</td>
</tr>
<tr>
<td>SNx</td>
<td>10</td>
<td>449 ± 30*</td>
<td>4.58 ± 0.45*</td>
<td>8.11 ± 0.54*</td>
<td>122.50 ± 15.78*</td>
<td>13.43 ± 0.61*</td>
</tr>
<tr>
<td>SNx + PFD</td>
<td>10</td>
<td>461 ± 31</td>
<td>4.07 ± 0.11</td>
<td>5.34 ± 0.97</td>
<td>99.80 ± 9.55†</td>
<td>10.43 ± 0.79†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10. SNx, 5/6 nephrectomy; PFD, pirfenidone; BW, body weight; KW/BW, left kidney weight per body weight; CRP, C-reactive protein; SCR, serum creatinine; BUN, blood urea nitrogen. *P < 0.05 vs. sham; †P < 0.05 vs. SNx rats.
pirfenidone significantly improved the elevated proteinuria. During the period of observation, this drug consistently inhibited the progression of proteinuria. Urinary excretion of NAG, a lysosomal enzyme found in tubular epithelial cells, is one indicator of the presence of lesions in the renal tubule. NAG activity was elevated in the SNx rats from the second week, and pirfenidone caused a significant degree of decrease in this activity (Fig. 1B). SBP was elevated from the sixth week in the SNx rats, and no significant difference was found after pirfenidone treatment (Fig. 1C).

Effects of pirfenidone on pathological change in SNx rats. To investigate the effect of pirfenidone on renal fibrosis, histological studies were performed. In the SNx rats, significant glomerulosclerosis and tubulointerstitial lesions were observed (Fig. 2H). Renal tubules around the glomeruli were severely degenerated, and the tubular lumen was dilated. Remarkable infiltration of inflammatory cells was observed in the cortical interstitium (Fig. 2B), and comparatively severe fibrosis was present (Fig. 2E). The effect of pirfenidone was evident. It attenuated interstitial fibrosis in the cortex (Fig. 2F) and decreased the infiltration of inflammatory cells in the cortical interstitium (Fig. 2C). It significantly reduced the scores of tubulointerstitial fibrosis and tubulointerstitial inflammation (Table 3). Glomerular alterations, such as sclerosis, were also improved (Fig. 2I). Pirfenidone significantly reduced the scores of glomerulosclerosis (Table 2).

Effects of pirfenidone on fibrotic markers in SNx rats. Protein expression of TGF-β1, CTGF, α-SMA, fibronectin, and FSP-1 was increased in the SNx rats. Pirfenidone treatment significantly decreased their expression (Fig. 3).

Effects of pirfenidone on tubulointerstitial and glomerular macrophage infiltration. As shown in Table 4, the number of macrophages (CD68+) was higher in the SNx rats (Fig. 4B) than in the sham-operated group (Fig. 4A). In the rats treated with pirfenidone, infiltrating macrophages in the tubulointerstitium and glomeruli were significantly reduced (Fig. 4C). Tubulointerstitial and glomerular macrophages with M1 (CCR7+) and M2 (CD163+) phenotypes were also increased in the SNx rats (Fig. 4, E and H). The number of M1 and M2 macrophages was significantly lower after pirfenidone treatment (Fig. 4, F and I).

Effects of pirfenidone on chemokines including MCP-1 and MIP-1α expression. The mRNA and protein expressions of MCP-1 and MIP-1α were increased in the SNx rats. Pirfenidone treatment significantly decreased their expression (Fig. 5).

Effects of pirfenidone on inflammatory markers expressed by M1 and M2 macrophage phenotypes. The TNF-α, IL-6, and iNOS expressed by M1 macrophages were increased in the SNx rats, and pirfenidone significantly reduced their expression at mRNA and protein levels. Conversely, pirfenidone significantly decreased mRNA and protein of arginase-1 and dectin-1 expressed by M2 macrophages compared with that in the SNx rats (Fig. 6).

Effects of pirfenidone on M2 macrophage subtypes. CD206 expressed by M2a and M2c cells was significantly reduced by pirfenidone at mRNA and protein levels. Pirfenidone also significantly decreased mRNA and protein levels of CD86 expressed by M2b cells (Fig. 7).

Correlation between macrophage infiltration and renal fibrosis. As shown in Fig. 8, no significant correlations between M1 macrophage infiltration and glomerulosclerosis or tubulointerstitial fibrosis were observed. However, a positive correlation was observed between M2 macrophage infiltration and tubulointerstitial fibrosis. Moreover, a negative correlation was observed between M2 macrophage infiltration and glomerulosclerosis.
stitial fibrosis were found \((r = 0.271, P = 0.449 \) for glomerulosclerosis; \(r = 0.231, P = 0.520 \) for tubulointerstitial fibrosis). However, significant correlations were observed between M2 macrophage infiltration and both glomerulosclerosis and tubulointerstitial fibrosis \((r = 0.724, P = 0.05 \) for glomerulosclerosis; \(r = 0.758, P = 0.05 \) for tubulointerstitial fibrosis).

DISCUSSION

In this study, we evaluated the effect of pirfenidone in a CKD murine model. It was demonstrated that pirfenidone could reduce plasma creatinine and blood urea nitrogen. These results are in accordance with previous experimental and clinical studies, which have demonstrated that pirfenidone improves renal functions (14, 34). Persistent elevation of CRP, a widely used inflammatory marker, in CKD patients predicted a worse survival (24). In a randomized controlled trial of patients with moderate CKD, lowered CRP was associated with a delay in renal progression (7). In this study, pirfenidone reduced the level of rat serum CRP in SNx rats, which suggests a potential anti-inflammatory effect on circulating inflammatory markers. We also found that pirfenidone decreased urinary protein excretion, whereas it did not influence SBP, which is not

Table 3. Effect of pirfenidone on glomerular and tubulointerstitial morphology

<table>
<thead>
<tr>
<th>Index</th>
<th>Sham</th>
<th>SNx</th>
<th>SNx + PFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulosclerosis score</td>
<td>0.07 ± 0.04</td>
<td>3.19 ± 0.18*</td>
<td>2.04 ± 0.29†</td>
</tr>
<tr>
<td>Tubulointerstitial fibrosis score</td>
<td>0.00 ± 0.00</td>
<td>62.70 ± 8.07*</td>
<td>31.10 ± 10.63†</td>
</tr>
<tr>
<td>Tubulointerstitial inflammation score</td>
<td>0.00 ± 0.00</td>
<td>148.00 ± 35.02*</td>
<td>59.70 ± 17.01†</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 10 \). *\( P < 0.05 \) vs. sham; †\( P < 0.05 \) vs. SNx rats.
similar as what Takakuta et al. (36) found. The difference might be related with the different application of the drug. In the study of Takakuta et al., they administrated pirfenidone by putting the drug in the chow, which may affect the real intake amount of the drug (32). While in our study, we treated the rats by gavage. In another animal model (antiglomerular basement membrane glomerulonephritis rat model), Leh et al. (14) also found pirfenidone to have no effect on blood pressure. This was also confirmed in patients with focal segmental glomerulosclerosis (4), suggesting that the antifibrotic properties of pirfenidone may not be related with its antihypertensive effect.

The antifibrotic activity of pirfenidone has been tested in a variety of cellular and animal models of fibrosis and has been shown to have antifibrotic and antiproliferative properties (18). We found that pirfenidone significantly reduced expression of TGF-β1, α-SMA, CTGF, fibronectin, and FSP-1, which exhibit its antifibrotic properties. Previous studies also found that pirfenidone inhibit TGF-β, collagen I/IV, and fibronectin expressions in the same animal model (35, 36). However, its antifibrotic mechanisms are still not fully understood. Macrophages have traditionally been recognized as key players in renal fibrosis (37). Macrophage depletion significantly reduced interstitial fibrosis in a unilateral ureteral obstruction model, which suggests that macrophages are profibrotic in this model (9).

In this study, we observed the effect of pirfenidone on macrophage infiltration. The secretory profile of inflammatory macrophages mirrors what has been classified as an M1 macrophage by in vitro stimulation with lipopolysaccharide and other microbial products (20). M1 macrophages secrete a significant amount of proinflammatory cytokines, such as TNF-α and IL-6, and they cause a release of nitric oxide from L-arginine due to iNOS activity (22). M1 macrophages are potentially cytotoxic and may induce vascular injury or tubular injury. Studies have suggested that M1-like cells are common in the early phases of repair (5). CCR7 is a surface marker indicative of an M1 phenotype (3). We found that renal infiltration of M1 was markedly increased in the SNx rats and the pirfenidone-treated SNx rats had a significantly lower infiltration of M1 and lower level of inflammatory mediators, including TNF-α, IL-6, and iNOS, which suggests that pirfenidone takes effect in the earlier period after inflammation initiation. The inhibition of proinflammatory mediators ameliorated the renal inflammatory microenvironment, which alleviated tissue injury. Persistent kidney inflammation is thought to contribute to the development of renal fibrosis with functional impairment. Thus pirfenidone’s antifibrotic mechanisms may be partly dependent on its anti-inflammatory effects by the reduction of proinflammatory mediators released by M1 macrophages.

Chronic kidney injuries are more often affected by renal cell apoptosis, in which pentraxin-mediated phagocytosis of apoptotic cells and homeostatic mediators promote macrophage polarization toward an anti-inflammatory M2-like phenotype (17). Differing from M1 macrophages, M2 macrophages do not

Fig. 3. Effects of PFD on fibrotic markers including transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF), α-smooth muscle actin (α-SMA), fibronectin, and fibroblast-specific protein-1 (FSP-1) protein expression. A: representative Western blotting analysis of TGF-β1, CTGF, α-SMA, fibronectin, and FSP-1; β-actin was used as an internal control. B: quantification of their protein expression. Values are represented as means ± SE; n = 6. *P < 0.05 vs. sham; #P < 0.05 vs. SNx rats.
express iNOS but express high levels of arginase-1 and dectin-1. This expression induces the production of proline in the metabolic pathway of nitric oxide, which stimulates cell growth, collagen formation, and tissue repair (10). M2-like cells with less proinflammatory cytokines and elevated markers of alternative activation, including arginase-1 and dectin-1 are common in late repair (5). M2 macrophages suppress inflammatory responses and adaptive Th1-immunity but promote a Th2 response, which has been linked to fibrogenesis and tissue remodeling (42). CD163 is surface marker representative of an M2 phenotype (3), and pirfenidone also reduced M2 macrophages and lowered the protein levels of arginase-1 and dectin-1. In a study of 4A3-deficient mice with Alport syndrome, the progression of Alport syndrome was associated with significant M2 macrophage infiltrates (33). Other murine CKD models also demonstrated that M2 macrophage infiltrate inter-

### Table 4. Effect of pirfenidone on tubulointerstitial and glomerular staining of macrophages

<table>
<thead>
<tr>
<th>Group</th>
<th>Tubulointerstitial</th>
<th>Glomerular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD68</td>
<td>CCR7</td>
</tr>
<tr>
<td>Sham</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>SNx</td>
<td>36.10 ± 6.67*</td>
<td>12.40 ± 1.78*</td>
</tr>
<tr>
<td>SNx + PFD</td>
<td>21.60 ± 4.06†</td>
<td>7.30 ± 1.95†</td>
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</table>

Values are means ± SE; n = 10. *P < 0.05 vs. sham; †P < 0.05 vs. SNx rats.

Fig. 4. Renal tissues obtained from sham-operated (A, D, and G), SNx (B, E, and H), and PFD-treated rats (C, F, and I). Representative examples of immunostaining in glomerular and tubulointerstitial areas: A–C: typical stains for CD68-positive macrophages. D–F: typical stains for CCR7-positive (M1) macrophages. G–I: typical stains for CD163-positive (M2) macrophages. Original magnification: ×200.
MCP-1 and MIP-1α infiltration

PMFs are a prominent source of TGF-β1, which promotes inflammation, chemotaxis, and excessive extracellular matrix deposition. Thus pirfenidone’s antifibrotic properties may be partly attributable to the inhibition of M2 macrophage infiltration.

M2 macrophages have been subdivided further into M2a, M2b, and M2c cells. CD163 is a common marker among all the subtype of M2 macrophages while CD206 is the marker only for M2a and M2c macrophages (6). M2a cells promote the production of extracellular matrix and tissue remodeling and have been characterized as profibrotic, and M2c macrophages also play a role in matrix deposition and tissue remodeling (29). Pirfenidone decreased expressions of CD206, suggesting its inhibition in M2a and M2c cells. CD86 appears to be a relatively specific marker for the M2b state (25), and its expression was also downregulated after pirfenidone, signifying that pirfenidone also inhibited M2b subtypes, which may sustain chronic inflammatory microenvironment during later period of renal fibrosis. In vivo microenvironment is far more complicated, and the effect of pirfenidone on shift among M2 macrophage subtypes polarization needs next investigation.

It is likely that proinflammatory and profibrotic macrophages coexist and drive both inflammation-mediated loss of renal cells and fibrogenesis (1). The role of multifaceted macrophages in the development of renal fibrosis may change in different stages of renal fibrosis. In this study, no significant correlations were observed between M1 macrophage infiltration and renal fibrosis. However, there are significant correlations between M2 macrophage infiltration and glomerulosclerosis or tubulointerstitial fibrosis. The nephrectomized rat is an animal model that represents CKD in the later period. No evident pathological changes were found in the earlier period after the model was established. Thus this study suggests that M1 and M2 macrophages exert their effects in the earlier and later period of renal fibrosis. Pirfenidone significantly ameliorated renal fibrosis accompanied by the inhibition of macrophage infiltration, which strongly suggests that the effectiveness of this drug in earlier and later periods of renal fibrosis.

Chemokines, such as MCP-1 (CCL2) and MIP-1α (CCL3), attract monocytes to the damaged sites, which likely results in differentiation of monocytes into macrophages with different phenotypes (19). Our previous study demonstrated that the urinary levels of MCP-1 and MIP-1α were significantly decreased in CKD patients after irbesartan treatment (26). We found that pirfenidone reduced the level of MCP-1 and MIP-1α significantly. In a bleomycin-induced murine pulmonary fibrosis model, pirfenidone also suppressed MCP-1 levels induced by bleomycin (28). An in vitro study further showed that pirfenidone inhibited T-cell receptor-induced production of multiple proinflammatory chemokines, including IP-10, MIP-1, and Mig, which suggests its inhibition of inflammatory cell recruitment (39). The functional phenotype of macrophages depends on the renal microenvironment, which changes during the pathological process (15). Thus pirfenidone may suppress macrophage infiltration and influence its phenotype by reducing chemokines.

In summary, our study demonstrates that pirfenidone inhibited both M1 and M2 infiltration in 5/6 nephrectomized rats. Inhibition of macrophage infiltration by pirfenidone may be involved in its therapeutic effects, which is at least beneficial for the treatment of renal fibrosis. This finding provides new
insight into the role of pirfenidone in retarding the progression of CKD.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.-F.C., H.F.N., M.-M.P., H.L., M.X., and M.-H.Z. performed experiments; J.-F.C. and H.F.N. analyzed data; J.-F.C., H.L., and B.-C.L. interpreted results of experiments; J.-F.C. prepared figures; J.-F.C. and H.F.N. drafted manuscript; J.-F.C. approved final version of manuscript; B.-C.L. conceived the hypothesis and designed of research; B.-C.L. edited and revised manuscript.

REFERENCES


**Fig. 6.** Effects of PFD on M1 macrophages correlated with TNF-α, IL-6, and nitric oxide synthases-2 (iNOS) protein expression, and M2 macrophages correlated with arginase-1 and dectin-1 expression. A: quantification of mRNA of M1 and M2 macrophage correlated markers; GAPDH was used as control. B: representative Western blotting analysis of M1 and M2 macrophage correlated markers; β-actin was used as control. C: quantification of protein of M1 and M2 macrophage correlated markers. Values were represented as means ± SE; n = 6. *P < 0.05 vs. sham; #P < 0.05 vs. SNx rats.


